

### Amendments to the specification

The amendments to the specification at pages 19 and 34 correct obvious typographical errors and do not add new matter.

### Objection to specification

Applicants have been unable to find in the M.P.E.P. the “form paragraph” cited in the Office Action with regard to disclosing hyperlinks to the Internet. To our understanding, reference to a world wide web (www) site is not improper. Clarification is requested. For the time being, the specification has not been amended on page 15, as was suggested in the Office Action of August 17, 2000.

### Sequence Listing

a) Page 19, lines 26-31 show only two sequences: a peptide having 20 residues, which begins D-D-D and ends H-H-H, and which is written on two lines (SEQ ID NO: 26); and a corresponding oligonucleotide having 72 bases, which is also written on two lines (SEQ ID NO: 25). Lines 26-28 of the specification have been amended to clarify the relative spacing of the amino acid residues and the corresponding triplet codons in the first lines of each of those sequences.

b) Figure 6 refers to a polypeptide (L19) comprising three sequences: a VH region (SEQ ID NO: 19), a linker (SEQ ID NO: 20) and a VL region (SEQ ID NO: 21). This is clearly described in the Detailed Description of the Drawings (see, *e.g.*, page 16, lines 10-11). The legend for Figure 6 starting at page 14, line 2 has been amended for clarity.

### Content of the Specification

The Brief Description of the Drawings, especially when considered in light of the Detailed Description of the Drawings that immediately follows it, and the labels found in the drawings, themselves, is clear and requires no amendment. With regard to the abbreviations in Figure 7, these terms are well-known in the art and are readily understood by one of ordinary skill in the art. For example, “VEGF” is “vascular endothelial growth factor” (see, *e.g.*, the description of Fig. 7 at page 27, lines 1-2 of the specification); and “PMA” is “phorbol 12-myristate 13-acetate” (see, *e.g.*, page 27, lines 2-3).

Claim amendments/ rejections under 35 U.S.C. §101 and §112, second paragraph

Claims 19-34 have been amended to place them in a form more consistent with U.S. patent practice, thereby obviating the rejections under 35 U.S.C. §101. Applicants disagree that the terms “improved affinity to said ED-B epitope” or “characteristic epitope” render the claims indefinite. Nevertheless, in an effort to expedite prosecution, the claims have been amended to clarify their meaning. The scope of the claims is unaltered.

Applicants maintain that original claim 19 is not unclear; the “diagnosis” and “therapy” methods require the same procedure: administering the recited antibody to patient in need thereof. Nevertheless, in an effort to expedite prosecution, claim 19 has been amended to recite a method of diagnosis, and new claim 40 has been added.

The term “ED-B” has not been amended. This term is an art-recognized term denoting this oncofetal domain of fibronectin. See, *e.g.*, the specification at page 1, lines 29-32, for a description of this fibronectin domain. One of skill in the art would recognize this term without the need of further explanation.

With regard to paragraph 15 of the Office Action, claim 31 further limits claim 30 in that it recites treating a particular type of angiogenesis-related pathology, an ocular disorder.

With regard to paragraph 17 of the Office Action, applicants are unaware of any legal requirement that the claims must recite “providing a conjugate” or “a correlation step with the preamble of the claim.” Under 35 U.S.C. §112, it is the applicant who decides what is being claimed, not the Patent Office.

New claims have been added which recite, *e.g.*, that the affinity of the antibody for the ED-B domain is “in the subnanomolar range” (*e.g.*, claims 36-44 and 46) or that the affinity is about 54 pM (*e.g.*, claim 45) or about 0.05 nM (*e.g.*, claim 47). Support for the recitation of “subnanomolar affinity” is found in the specification, *e.g.*, at page 1, lines 5-6 and at page 4, lines 27-28. An antibody which is exemplified in the specification - antibody L19 - exhibits a  $K_d$  of only 0.054 nM, within the recited range of “in the subnanomolar range.”

Rejections over alleged prior art

The Carnemolla and Neri (WO97/45544) references (cited in paragraphs 19 and 20 of the Office Action) have similar disclosures of anti ED-B antibodies generated by recombinant

methods. Neither reference anticipates the instant claims, at least because the references do not disclose an antibody which exhibits “high affinity” for the ED-B domain. The references disclose antibodies which bind to ED-B with affinities of, *e.g.*, 53 nM or 1.1 nM; neither of these affinities qualifies as “high affinity.” They certainly do not exhibit an affinity in the “subnanomolar range” or “about 0.05 or 0.054 nM” as recited, *e.g.*, in dependent claims 36, 45 or 47. Neither reference discloses all the material elements of the claims; therefore, neither reference is anticipatory.

The Mariani reference (cited in paragraph 21 of the Office Action) does not anticipate the instant claims. Mariani is drawn to the “BC-1” antibody, which recognizes domain 7 of fibronectin (which is adjacent to the ED-B domain), not the ED-B domain. As is noted in the instant specification, for example at the paragraph beginning at page 3, line 29, with regard to the BC-1 antibody (which is also discussed in Carnemolla *et al.* 1992, *J. Biol. Chem.* 267, 24689-92), “the BC1 antibody ... recognizes an epitope on domain 7 of FN [fibronectin], but not on the ED-B domain, which is cryptic in the presence of the ED-B domain of fibronectin. ... Therefore, the BC-1 antibody and the antibodies of the present invention show different reactivity.” BC-1 is clearly different from the instantly claimed antibody, and thus does not anticipate it.

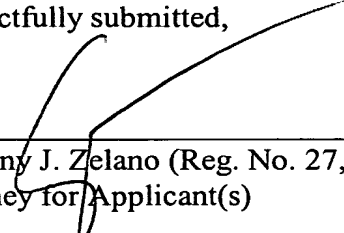
The two Thorpe references (cited in paragraphs 22 and 23 of the Office Action) not do anticipate the instant claims, at least because they do not disclose an antibody specific for ED-B. At best, they disclose an antibody which recognizes “a tumor associated fibronectin isoform,” “*e.g.*, as recognized by the MAb BC-1.” (see, *e.g.*, the ‘289 patent at col. 8, lines 7-11). As was discussed above in relation to the Mariani reference, the BC-1 antibody recognizes domain 7 of fibronectin, not the ED-B domain.

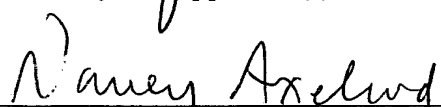
As for the obviousness rejections (paragraphs 25-27 of the Office Action), none of the references of record, taken separately or together, suggests or discloses that in the present context (*i.e.*, a conjugate comprising an antibody specific for ED-B), it would be desirable to generate a conjugate which comprises an antibody with a specific, high affinity for ED-B, let alone one with an affinity in the subnanomolar range. That is, they do not provide motivation to do so. The attached paper by Viti *et al.* [(1999). *Cancer Research* 59, 347-352], whose authors overlap with the instant inventors, demonstrates the desirability of high affinity for the angiogenic properties of the antibody.

In view of the above amendments and arguments, the application is believed to be in condition for allowance, which action is respectfully requested.

Respectfully submitted,

By:

  
\_\_\_\_\_  
Anthony J. Zelano (Reg. No. 27,969)  
Attorney for Applicant(s)

  
\_\_\_\_\_  
Nancy J. Axelrod (Reg. No. 44,014)  
Patent Agent

MILLEN WHITE ZELANO & BRANIGAN  
Arlington Courthouse Plaza 1  
2200 Clarendon Blvd., Suite 1400  
Arlington, VA 22201  
Direct Dial: (703) 812-5304  
Internet address: axelrod@mwzb.com

**Filed: April 17, 2001**

K:\PAT\NOTAR 1 P2 (see also SCH1733P2)\reply.doc

## MARKED-UP VERSION

### IN THE SPECIFICATION:

Fig. 6 shows amino acid sequence of L19 (VH, linker and VL, SEQ ID NOS. 19-21, respectively).

Not1            D   D   D   S   D   D   D   Y   K   D   D  
5' - GCG GCC GCA GAT GAC GAT TCC GAC GAT GAC TAC AAG GAC GAC --

Table 1:

Sequences of selected anti-ED-B antibody clones

		VH chain			VL chain		
Clone	31-33*	50-54*	95-98*	32*	50*	91-96*	
A2	SYA	AISGSG (SEQ ID NO. 27)	GLSI (SEQ ID NO. 29)	Y	G	NGWYPW (SEQ ID NO. 32)	
G4	SYA	AISGSG (SEQ ID NO. 27)	SFSF (SEQ ID NO. 30)	Y	G	GGWLPY (SEQ ID NO. 33)	
E1	SYA	AISGSG (SEQ ID NO. 27)	<del>FPFY</del> <u>PFPY</u> (SEQ ID NO. 31)	Y	G	TGRIPP (SEQ ID NO. 34)	
H10	SFS	SIRGSS (SEQ ID NO. 28)	<del>FPFY</del> <u>PFPY</u> (SEQ ID NO. 31)	Y	G	TGRIPP (SEQ ID NO. 34)	
L19	SFS	SIRGSS (SEQ ID NO. 28)	<del>FPFY</del> <u>PFPY</u> (SEQ ID NO. 31)	Y	Y	TGRIPP (SEQ ID NO. 34)	

### IN THE CLAIMS:

19. (Amended) A Method method for diagnosis and therapy of tumours diagnosing a tumor and diseases or disease characterized by vascular proliferation ~~wherein an antibody, comprising administering to a patient in need of such diagnosis an antibody with specific, high affinity for a characteristic epitope of the ED-B domain of fibronectin, said antibody having improved affinity to said ED-B domain, is used.~~

20. (Amended) A Conjugates conjugate comprising an antibody ~~according to Claim 1~~ with a specific, high affinity for the ED-B domain of fibronectin and a molecule ~~capable of inducing which induces~~ blood coagulation and/or blood vessel occlusion.

21. (Amended) A Conjugates conjugate according to claim 20 wherein the molecule ~~capable of inducing which induces~~ blood coagulation and/or blood vessel occlusion is a photoactive molecule.

22. (Amended) ~~A Conjugates~~ conjugate according to claim 21 wherein the photoactive molecule is a photosensitizer.

23. (Amended) ~~A Conjugates~~ conjugate according to claim 22 wherein the photosensitizer absorbs at a wavelength above 600 nm.

24. (Amended) ~~A Conjugates~~ conjugate according to claim 22 wherein the photosensitizer is a ~~derivative of tin (IV) chlorine e6~~ e6 molecule.

25. (Amended) ~~A Conjugates~~ conjugate according to claim 20 wherein the molecule ~~capable of inducing~~ which induces blood coagulation and/or blood vessel occlusion is a radionuclide.

26. (Amended) ~~A Conjugates~~ conjugate according to claim 25 wherein the radionuclide is an  $\alpha$ - or  $\beta$ - emitting radionuclide.

27. (Amended) ~~A Conjugates~~ conjugate according to claim 26 wherein the  $\alpha$ -emitting radionuclide is astatine-211, bismuth-212, or bismuth-213.

28. (Amended) ~~A Conjugates~~ conjugate according to claim 20 wherein the molecule ~~which induces~~ capable of inducing blood coagulation and/or blood vessel occlusion is ~~represented by~~ comprises a photosensitizer and a radionuclide.

29. (Amended) ~~A Method~~ method for the treatment of an angiogenesis-related ~~pathologies wherein a pathology, comprising administering to a patient in need thereof a~~ conjugate according to claim 20 is injected.

30. (Amended) ~~A Method~~ method for the treatment of an angiogenesis-related ~~pathologies wherein a pathology, comprising administering to a patient in need thereof a~~ conjugate according to claim 22 is injected, followed by irradiation.

31. (Amended) ~~A Method~~ method according to claim 30 wherein the angiogenesis-related pathology is an ~~treated is caused by or associated with~~ ocular angiogenesis disorder.

32. (Amended) ~~A Method~~ method for the treatment of an angiogenesis-related ~~pathologies wherein a pathology, comprising administering to a patient in need thereof a~~ conjugate according to claim 25 is injected.

33. (Amended) ~~A Method~~ method according to claim 32 wherein the radionuclide is astatine-211.

34. (Amended) ~~A Method~~ method for the treatment of an angiogenesis-related ~~pathologies wherein a pathology, comprising administering to a patient in need thereof a~~ conjugate according to claim 28 ~~is injected~~.

Claims 36-48 have been added; therefore, no "marked-up" version is necessary.

## Increased Binding Affinity and Valence of Recombinant Antibody Fragments Lead to Improved Targeting of Tumoral Angiogenesis<sup>1</sup>

Francesca Viti, Lorenzo Tarli, Leonardo Giovannoni, Luciano Zardi, and Dario Neri<sup>2</sup>

*Institut für Molekularbiologie und Biophysik, ETH Hönggerberg, CH-8093 Zürich, Switzerland [F. V., L. T., D. N.]; Dipartimento di Biologia Molecolare, Sezione di Biochimica, Università di Siena, 53100 Siena, Italy [L. G.]; and Istituto Nazionale per la Ricerca sul Cancro/Centro di Biotecnologie Avanzate, 16132 Genova, Italy [L. Z.]*

### ABSTRACT

The formation of new blood vessels (angiogenesis) is an important step in tumor progression. Molecules capable of selectively targeting markers of angiogenesis may offer opportunities for the *in vivo* imaging of aggressive tumors and for the delivery of toxic agents to the tumoral vasculature. Using antibody phage display libraries and combinatorial mutagenesis, we isolated single-chain Fv antibody fragments, which recognize with different affinities the same epitope of the ED-B domain of fibronectin, a marker of angiogenesis. Two single-chain Fv fragments, E1 and L19, with dissociation constants of 41 nM and 0.054 nM, respectively, were investigated for their ability to target F9 murine teratocarcinoma grafted s.c. in nude mice when injected i.v. in either monomeric or homodimeric form (*M*, 27,000 and 54,000, respectively). Biodistribution studies, performed at two time points (4 h and 24 h) with radiolabeled samples, showed that the higher affinity antibody targets the tumor significantly better than the lower affinity one, in terms both of tumor:organ ratios and of the amounts of antibody delivered to the tumor. In particular, more than 20% of the injected dose of dimeric L19 accumulated per gram of tumor at 4 h; the tumor:organ ratios at 4 h and 24 h were in the (2.1–8.6):1 and (10.3–29.4):1 range, respectively. This study demonstrates that, although vasculature represents only a small fraction of the total tumor mass, anti-ED-B antibodies can selectively target tumors *in vivo* and that this process is particularly efficient if very high-affinity binders are used.

### INTRODUCTION

Most aggressive solid tumors cannot grow beyond a certain mass without the formation of new blood vessels (angiogenesis), and a correlation between microvessel density and tumor invasiveness has been reported (1). Abs<sup>3</sup> or other suitable molecules that are capable of selectively targeting markers of angiogenesis would create clinical opportunities for the diagnosis and therapy of tumors and other diseases characterized by vascular proliferation (2–8).

The targeting of angiogenesis is attractive for a number of reasons. Because tumor growth and metastatic spread depend on angiogenesis, the selective delivery of toxic agents to new-forming blood vessels should offer a therapeutic benefit (2–8). Unlike tumor cells, endothelial cells do not mutate, and resistance is, therefore, less likely to develop. It should be possible to use a single reagent, specific for angiogenesis, for the selective detection and/or ablation of different types of tumor. Furthermore, markers on new-forming blood vessels should be readily accessible to binding molecules (e.g., Abs) injected i.v. and should overcome the well-recognized problem of slow tumor penetration of targeting agents specific for markers on the membrane of tumor cells (9–11).

Research into antiangiogenic therapeutic strategies has mainly fo-

cused on the search for inhibitory molecules (1–3). Only few reports, with no quantitative biodistribution analyses, have indicated that it may indeed be possible to target angiogenesis *in vivo* (5, 12).

The ED-B domain of fibronectin, a sequence of 91 amino acids identical in mouse, rat, dog, and man, which is inserted by alternative splicing into the fibronectin molecule, specifically accumulates around neovascular structures (13–17) and could represent a target for molecular intervention. Indeed, we have recently shown with fluorescent techniques that anti-ED-B scFvs<sup>3</sup> (12) that are injected in tumor-bearing mice selectively accumulate in tumoral blood vessels.

These experiments, performed using Ab fragments with affinities in the low nanomolar range, demonstrated that it is possible to selectively target tumoral angiogenesis *in vivo*. They also raised the issue of whether Abs against markers of angiogenesis with further enhancement in binding affinity and/or valence would show an improved localization in tumoral neovasculature—an important parameter for the development of diagnostic and therapeutic applications.

Here we present the results of biodistribution studies in tumor-bearing mice performed with two radiolabeled Abs directed against the same epitope of the ED-B domain of fibronectin and differing in affinity by 760-fold. We quantitatively investigated whether binding affinity influences tumor targeting *in vivo* when Abs are injected i.v. as purified monomers or homodimers.

### MATERIALS AND METHODS

**Production of Anti-ED-B Affinity Mutants.** The isolation of the E1 and L19 Abs has been described previously (18). E1 is a scFv binding to the ED-B domain of fibronectin that is isolated from a synthetic human phage display Ab library. L19, a mutant of E1 with 760-fold improved affinity, differs from the parental Ab by eight mutations introduced in hypervariable loops (European Molecular Biology Laboratory accession no. AJ006113; Ref. 18).

The genes coding for scFv(E1) and scFv(L19) were subcloned into *NcoI*/*NotI* digested pDN5 (19) according to standard techniques (20). The corresponding Ab fragments, carrying a *myc* tag epitope at the COOH terminus (21), were expressed from TG1 *Escherichia coli* cells harboring the appropriate plasmids and were affinity-purified from bacterial supernatants using an ED-B-Sepharose column, as described previously (12).

Native PAGE (19) and gel filtration analysis of purified scFvs showed that E1 and L19 consist of a monomeric and a dimeric fraction, which were quantitatively separated and recovered using a Superdex 75 HR 10/30 column (Pharmacia, Uppsala, Sweden). The stability of the purified monomer and dimer was checked by native gel electrophoresis with Homogenous 12.5% gels (Pharmacia) by using a Phast System (Pharmacia) as described previously (19).

The stability of E1 and L19 in serum was evaluated by incubating the Ab in FCS at 37°C for different time periods (0–4 h) and analyzing the Ab reactivity in the resulting mixture by ELISA as described previously (14), applying biotinylated ED-B onto streptavidin-coated ELISA plates (Boehringer, Mannheim, Germany).

**Epitope Retention Assay.** The ability of L19 to compete for the binding of E1 to a common epitope on the ED-B domain of fibronectin was investigated by real-time interaction analysis with surface plasmon resonance detection, using a BIAcore 1000 instrument (Pharmacia), according to established protocols (12, 22). Biotinylated ED-B (12) was applied to streptavidin-coated microsensor chips until the resulting surface bound ~ 1000 RUs of scFv fragments. Binding studies were performed using 1  $\mu$ M purified scFv solutions in PBS (50 mM phosphate buffer (pH 7.2)–100 mM NaCl), with a 5- $\mu$ L/min flow.

Received 7/27/98; accepted 11/11/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup>Supported by an internal Grant of the ETH Zürich (to D. N.), by the Stiftung zur Krebsbekämpfung (to D. N.), and by the Associazione Italiana Ricerca sul Cancro (to L. Z.).

<sup>2</sup>To whom requests for reprints should be addressed, at Institut für Molekularbiologie und Biophysik, ETH Hönggerberg, CH-8093 Zürich, Switzerland. E-mail: dario@mol.biol.ethz.ch.

<sup>3</sup>The abbreviations used are: Ab, antibody; scFv, single-chain Fv Ab fragment; T:O, tumor:organ; %ID, percent injected dose; RU, (BIAcore) resonance unit.



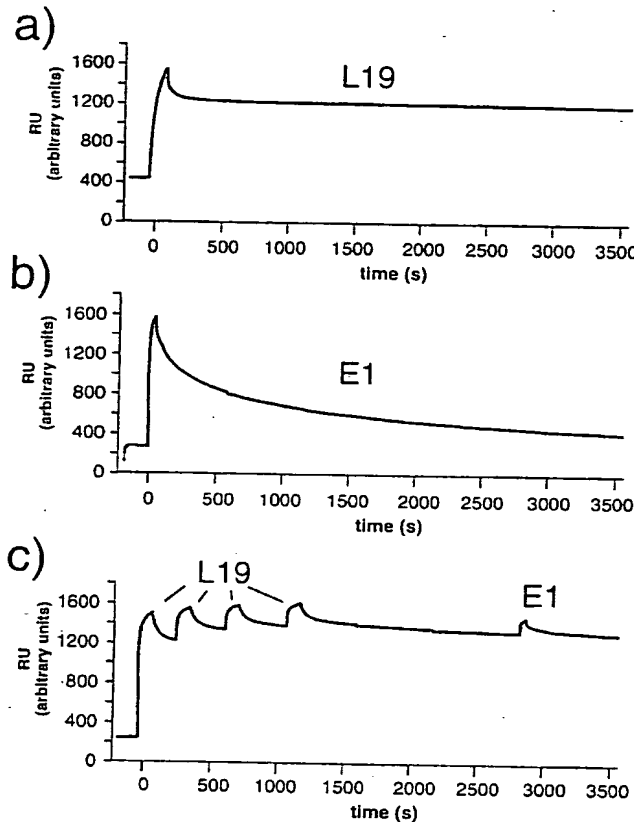


Fig. 1. Epitope retention assay of anti-ED-B Abs. The binding properties of anti-ED-B Abs L19 and E1 were investigated by real-time interaction analysis with surface plasmon resonance, using a BIAcore instrument and a microsensor chip coated with recombinant ED-B domain of fibronectin. L19 and E1 Abs were injected onto the microsensor chip either individually (a and b) or sequentially (c).

For studies of competitive binding to the same epitope of the ED-B domain, the higher affinity scFv(L19) was injected first until saturation of the microsensor chip was achieved, inasmuch as this Ab shows a flat dissociation profile.

Western blot analysis of Ab specificity was performed as described previously (17).

**Radiiodination.** Immediately after their chromatographic separation (see previous section), dimeric and monomeric forms of scFv(L19) were radiolabeled with  $^{125}\text{I}$  using the Iodogen method (Pierce, Rockford, IL). Briefly, 100–200  $\mu\text{g}$  of protein were combined with 100–200  $\mu\text{Ci}$  of  $^{125}\text{I}$ , followed by separation from unincorporated iodine using a PD-10 disposable gel filtration column (Pharmacia). Ab immunoreactivity after labeling was evaluated by loading an aliquot of radiolabeled sample onto 200  $\mu\text{l}$  of ED-B-Sepharose resin (capacity, >3 mg scFv/ml resin; Ref. 12) on a pasteur pipette, followed by radioactive counting of the flow-through and eluate fractions. Immunoreactivity, defined as the ratio between the counts of the eluted protein and the sum of the counts of the eluted and flow-through fractions, was 81% for monomeric L19, 92% for dimeric L19, 90% for monomeric E1, and 91% for dimeric E1.

The purity of radiolabeled samples was analyzed by reducing SDS-PAGE using Homogenous 20 Phast gels and a Phast System (Pharmacia), followed by autoradiography with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Native PAGE of the radiolabeled samples, performed using Homogenous 12.5 Phast gels, native buffer strips, and a Phast System (Pharmacia) followed by autoradiography (19), was performed to ensure that monomeric and dimeric Ab samples retained their oligomeric state after radiiodination.

**Biodistribution Studies.** Biodistribution studies were performed under a license (*Tumor Targeting*, Bewilligung 53/97) issued to D.N. by the Veteri-

näramt des Kantons Zürich. Nude mice (10–14 week-old CD1 nude mice, females) were obtained from LTK (Zurich, Switzerland). F9 murine teratocarcinomas were implanted s.c. as described previously (12, 17). When tumor size reached 100–300 mg, 1  $\mu\text{g}$  (1  $\mu\text{Ci}$ ) of scFv in 150  $\mu\text{l}$  of saline solution, radiolabeled on the same day, was injected i.v. Previous biodistribution experiments with anti-ED-B Abs and F9 teratocarcinoma had shown that the amount of scFv injected is well below the dose necessary to saturate the tumor antigen pool. In fact, at least 10  $\mu\text{g}$  of scFv per gram of tumor can selectively localize in F9 teratocarcinoma (12). Furthermore, biodistribution experiments performed with two radiolabeled Ab fragments directed against irrelevant antigens resulted in tumor:blood ratios of <1 at 1–24 h after injection.

Mice were killed at 4 and 24 h after injection, and organs were weighed and radioactively counted. Three animals were used for each time point. Targeting results of representative organs are expressed as %ID of Ab/g of tissue. The mean error and SE for each group of data were calculated, and T/O ratios were determined. Significance levels were calculated using Student's *t* test.

Immunohistochemical studies with cryostat sections of F9 murine teratocarcinoma were performed as described previously (17).

## RESULTS

**Ab Fragment.** We have recently described (18) the isolation of two human scFv Ab fragments, E1 and L19, with dissociation constants for the ED-B domain of fibronectin of 41 nM and 0.054 nM, respectively. L19 is an affinity-matured version of E1, obtained by combinatorial mutagenesis of judiciously selected residues in the hypervariable loops of E1 and selected using phage display technology (18, 23). The enhanced binding affinity of L19 is mainly due to a lower kinetic dissociation constant (18). Immunohistochemistry studies with tumor sections have demonstrated that both Abs recognize (ED-B)-containing fibronectin in vascular structures (18).

To assess how binding affinity influences tumor targeting properties, it is essential to examine Abs that recognize the same antigen and the same epitope with different affinity values (10). Previous experiments that were aimed at affinity-maturing recombinant Abs using phage display technology have shown that affinity mutants retain the epitope of the parental Ab (12, 24–28). To confirm that E1 and L19 recognize the same epitope, we studied their binding to the ED-B domain of fibronectin covalently immobilized on a BIAcore microsensor chip (12, 22) by real-time interaction analysis with surface plasmon resonance detection. Fig. 1, a and b, shows the BIAcore sensograms of scFv(L19) and scFv(E1) binding to ED-B. The two Abs did not bind to microsensor chips coated with irrelevant antigens (streptavidin, glutathione *S*-transferase, BSA, and hen egg lysozyme; data not shown). The different kinetic stability of the two Ab-antigen

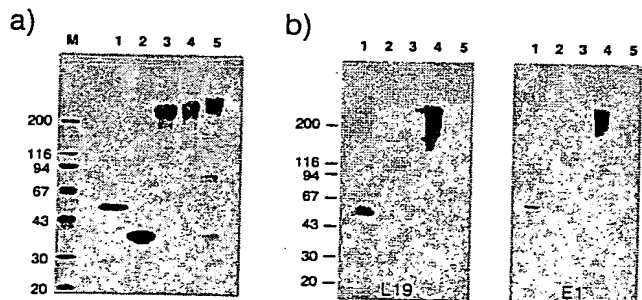


Fig. 2. Specificity of E1 and L19 Abs. Coomassie Blue-stained polyacrylamide gel (a) and immunoblot analysis, performed with L19 and E1 Ab (b), of proteins containing fibronectin or fibronectin-like repeats. Lanes: 1, recombinant fibronectin fragment 7B89 (containing repeats 7, ED-B, 8, and 9); 2, recombinant fibronectin fragment 7B9 (containing repeats 7, 8, and 9); 3, plasma fibronectin; 4, ED-B-containing fibronectin from WI38VA cells; 5, tenascin-C from SKMEL-28 cells. The preparation of these proteins was described previously (14). The molecular weight standards (M) are expressed in M, thousands.

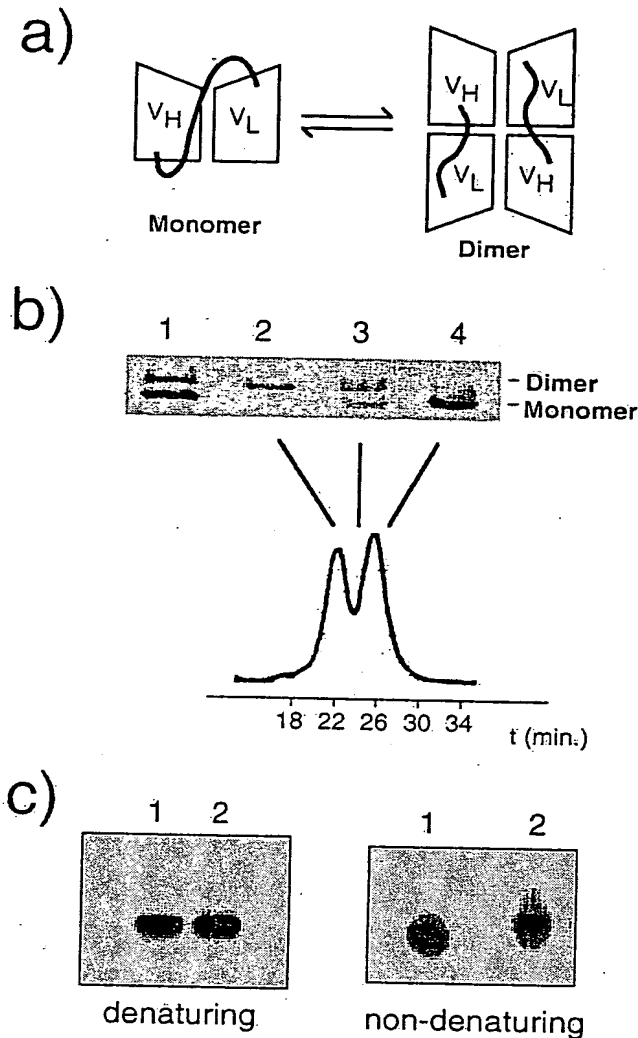


Fig. 3. Monomeric and dimeric fractions of scFvs. *a*, scFvs exist as a slowly interconverting equilibrium between a monomeric and a dimeric form. *b*, separation of monomeric and dimeric fractions of scFv(L19) by gel filtration on a Superdex 75 HR10/30 column and analysis of the purified fractions by nondenaturing gel electrophoresis. Lanes: 1, input; 2, dimer; 3, mixture of monomer and dimer; 4, monomer. *c*, autoradiogram of a SDS-PAGE and a nondenaturing PAGE of radiolabeled scFv(L19) monomer (Lane 1) and dimer (Lane 2). Essentially identical results were obtained for scFv(E1).

complexes can be observed in the portion of the sensogram during which the complexes are washed with a flow of buffered saline and are allowed to dissociate. We then saturated the ED-B-coated microsensor chip with four successive injections of L19 (amount of Ab bound, 1100 RUs) and tested whether E1 could still bind to the antigen at a comparable level of RUs (Fig. 1c). As expected, L19 inhibited the binding of E1 to the ED-B domain of fibronectin, which confirmed that the epitope recognized by the two Abs was retained during affinity maturation.

To further confirm that E1 and L19 retain the same binding specificity, immunoblot analysis was performed with fibronectins and fibronectin fragments. Both E1 and L19 reacted with ED-B-containing fibronectin and fibronectin fragments but not with plasma fibronectin, with fragments devoid of the ED-B domain, or with tena-

scin-C, a large multidomain protein containing several fibronectin-like modules (Fig. 2).

Most scFvs are a mixture of monomer and homodimer (Fig. 3a; Refs. 19, 29–31). Although the affinity of these molecular species toward a monomeric antigen are identical when measured in solution (32), the functional affinity (or “avidity”) of the dimer toward an immobilized antigen on tumoral vasculature can be significantly increased by rebinding effects and by the simultaneous binding of two antigens (32, 33). Furthermore, monomeric and dimeric scFvs have different sizes and pharmacokinetics (11, 34). It is, therefore, important that one works with purified Abs of a defined oligomeric state to study the role of binding affinity in the targeting of angiogenesis.

The monomeric and dimeric fractions of scFv(L19) and scFv(E1) were separated and quantitatively recovered by gel-filtration. A representative chromatogram is presented in Fig. 3b, together with the analysis of the recovered fractions performed by native PAGE. The recovered Ab fractions were labeled with <sup>125</sup>I using the Iodogen method (35). Ab immunoreactivity after radiolabeling ranged between 81% and 92% (see “Materials and Methods”). Purified monomeric and dimeric scFvs (Fig. 3c) incubated at 22°C for 5 days were shown by native PAGE to be stable before and after radiolabeling.

Both E1 and L19 monomeric and dimeric fractions showed comparable reactivity in immunohistochemistry with cryostat sections of F9 teratocarcinoma performed as described previously (Ref. 14; Fig. 4), suggesting that the Ab preparations conserve the same specificity toward new-forming blood vessels. We had previously shown that Ab affinity does not affect performance in immunohistochemistry (14).

**Biodistribution Studies.** We chose the F9 murine teratocarcinoma grafted s.c. in nude mice (12) as a syngeneic tumor model for evaluating Ab performance in the targeting of angiogenesis. Because the ED-B domain of fibronectin is identical in mouse and man, the results of our tumor targeting studies should be predictive of the Ab performance in humans. Fig. 4 shows that new-forming blood vessels of a section of F9 teratocarcinoma are strongly and specifically stained in red by scFv(L19) using immunohistochemical techniques. The neovasculature represents only a low percent of the total F9 tumoral mass.

Biodistribution studies at 4 h and 24 h were performed to examine the role of Ab affinity on the specificity and the degree of tumor retention. One  $\mu$ g of purified radiolabeled scFv (1  $\mu$ Ci) in either monomeric or dimeric form was administered to the mice by i.v. injection. The biodistribution results (Tables 1 and 2) revealed a correlation between the affinity and the %ID of radiolabeled Ab/g delivered to the tumor.

The tumor retention of both monomeric and dimeric L19 was significantly better than that of E1 (confidence level, >95% for each pairwise comparison of L19 and E1 at 4 h and 24 h). The amount of E1 monomer in the tumor decreased by more than 10-fold from 4 h to 24 h, compatibly with its more rapid dissociation from the antigen observed *in vitro* (Fig. 1b; Ref. 18). The time dependence of Ab retention in the tumor progressively improved for E1 dimer, L19 monomer, and L19 dimer. The latter Ab was retained in relatively large amounts in F9 teratocarcinoma (20.2 %ID/g at 4 h; 11.8 %ID/g at 24 h), the decrease in the %ID/g being due, at least in part, to the fact that the F9 teratocarcinoma doubles in size in 24–48 h. As previously described for different recombinant Abs (11, 34, 36), dimerization resulted in prolonged Ab retention in the blood and in larger %ID/g retained in tumor and in normal tissues, compared with the corresponding monomer.

The higher affinity L19 Ab was significantly superior to E1 also in terms of T:O ratios (confidence levels ranged between 80% and 99.99% for all of the pairwise comparisons of T:O ratios at 4 h and

Fig. 4. Immunohistochemistry of F9 murine teratocarcinoma. Immunohistochemical findings obtained with a cryostat section of F9 murine teratocarcinoma stained with scFv(L19) monomer. New-forming blood vessels are strongly stained in red by the monoclonal Ab. Cell nuclei are counterstained with Gill's hematoxylin. Note that the tumor vasculature represents only a small percentage of the total tumor mass. Scale bar, 10  $\mu$ m.

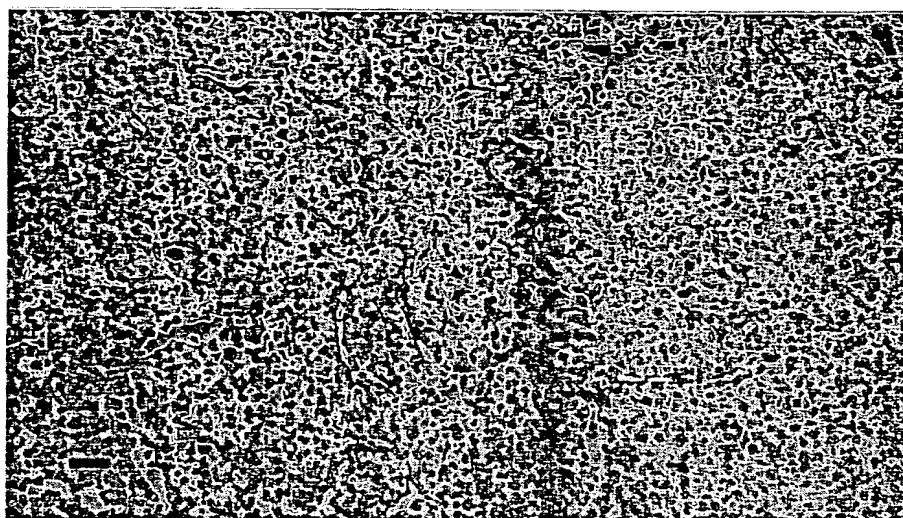


Table 1 Biodistribution of  $^{125}$ I-labeled E1 monomer and  $^{125}$ I-labeled E1 dimer in F9 tumor-bearing nude mice over 4 and 24 h

Iodinated Ab fragments were administered to nude mice bearing a s.c. grafted F9 murine teratocarcinoma (100–300 mg) by i.v. tail vein injection (1  $\mu$ g/mouse). Cohorts of three mice were killed 4 h and 24 h after injection, and organs were weighed and radioactively counted. Targeting results are expressed as %ID of  $^{125}$ I-labeled Ab retained per g of tissue. T:O ratios are also reported. Unless otherwise indicated, SEs were <22% of the indicated value.

Organs	scFv(E1) monomer				scFv(E1) dimer			
	4 h		24 h		4 h		24 h	
	%ID/g	T:O	%ID/g	T:O	%ID/g	T:O	%ID/g	T:O
Tumor	2.12 <sup>a</sup>		0.17		7.32		1.29	
Blood	1.55	1.36	0.07	2.42	5.89	1.24	0.62	2.04
Spleen	0.67	3.16	0.03	5.66	1.45	5.04	0.18	7.16
Liver	0.44 <sup>b</sup>	4.81	0.02	8.50	1.61 <sup>c</sup>	4.54	0.15	8.60
Lung	1.33	1.59	0.07	2.42	4.09	1.78	0.47	2.74
Kidney	2.98	0.71	0.14 <sup>d</sup>	1.21	5.89	1.24	0.49	2.63
Intestine	0.66 <sup>e</sup>	3.21	0.02	8.5	1.25	5.85	0.25	5.16

<sup>a</sup> SE = 31%.

<sup>b</sup> SE = 26%.

<sup>c</sup> SE = 28%.

<sup>d</sup> SE = 41%.

<sup>e</sup> SE = 33%.

Table 2 Biodistribution of  $^{125}$ I-labeled L19 monomer and  $^{125}$ I-labeled L19 dimer in F9 tumor-bearing nude mice over 4 and 24 h

Iodinated Ab fragments were administered to nude mice bearing a s.c. grafted F9 murine teratocarcinoma (100–300 mg) by i.v. tail vein injection (1  $\mu$ g/mouse). Cohorts of three mice were killed 4 h and 24 h after injection, and organs were weighed and radioactively counted. Targeting results are expressed as %ID of  $^{125}$ I-labeled Ab retained per g of tissue. T:O ratios are also reported. Unless otherwise indicated, SEs were <26% of the indicated value.

Organs	scFv(L19) monomer				scFv(L19) dimer			
	4 h		24 h		4 h		24 h	
	%ID/g	T:O	%ID/g	T:O	%ID/g	T:O	%ID/g	T:O
Tumor	5.08		1.69		20.23		11.78	
Blood	1.77	2.87	0.25 <sup>a</sup>	6.76	9.44 <sup>b</sup>	2.14	1.14	10.33
Spleen	0.68	7.47	0.18	9.38	2.40	8.42	0.40	29.45
Liver	0.53	9.58	0.09	18.70	2.34	8.64	0.46 <sup>c</sup>	25.60
Lung	1.33	3.81	0.17	9.94	5.06 <sup>d</sup>	3.99	0.75	15.70
Kidney	3.73	1.36	0.23	7.34	8.84	2.28	0.91	12.94
Intestine	0.69	7.36	0.14	12.07	2.93	6.90	0.76	15.50

<sup>a</sup> SE = 39%.

<sup>b</sup> SE = 42%.

<sup>c</sup> SE = 38%.

<sup>d</sup> SE = 28%.

24 h). Comparisons of the results obtained with monomeric and dimeric preparations revealed that T:O ratios were not significantly influenced by the Ab valence. However, T:O values for L19 dimer at 24 h were consistently better than those of the other Abs analyzed because of the longer Ab retention in the tumor.

## DISCUSSION

To our knowledge, this work represents the first quantitative examination of the effects of affinity and valence on the targeting of tumoral vasculature by recombinant Abs. We have investigated the

tumor targeting properties of two Abs, E1 and L19, in monomeric and homodimeric form, which are specific for the ED-B domain of fibronectin, a marker of angiogenesis (13–17). E1 and L19 bind to an identical epitope on the antigen with dissociation constants of 41 nM and 0.054 nM, respectively.

Our tumor model was an aggressive murine F9 teratocarcinoma tumor s.c. grafted in mice. It is generally believed that syngeneic models allow a better evaluation of Ab specificity than do models in which a human tumor marker is present in a human tumor xenografted in mice but is otherwise absent in other murine tissues. The situation with the ED-B domain of fibronectin is particularly favorable; because its amino acid sequence is identical in mouse and man, the results of our biodistribution experiments in mice may to some extent be predictive of the performance of the Abs in humans.

High-affinity L19—both monomer and dimer—target the tumoral neovasculature significantly better than their E1 counterparts (Tables 1 and 2). Tumor:blood ratios were already greater than 2 at 4 h after administration, suggesting the potential usefulness of L19 for the immunoscintigraphic detection of angiogenesis in patients—namely an application in which short-lived radionuclides such as  $^{99m}\text{Tc}$  or  $^{123}\text{I}$  have to be used to minimize exposure to radiation. Moreover, tumor: kidney and tumor:liver ratios, which are often problematic for intact monoclonal Abs and for some Ab fragments (37), were extremely favorable. Additional advantages are that recombinant human Abs: (a) can be expressed in large amounts in bacteria (38, 39) and yeast (40); (b) are not immunogenic; and (c) are easy to validate for clinical applications (41).

L19 dimer accumulates in the tumor in significantly larger amounts than does L19 monomer, both at 4 h and 24 h, given its longer half-life in blood. This observation was somewhat surprising considering the extremely high affinity of monomeric L19 and the good accessibility of its antigen, which is localized prevalently around new-forming blood vessels (Fig. 4). We can postulate two different explanations for the fact that targeting of the ED-B domain of fibronectin *in vivo* is a relatively slow process: (a) microcirculation in new-forming blood vessels could be impaired, preventing the Abs from rapidly reaching their target; or (b) the process of crossing the endothelial layer, which precedes the Abs' binding to the fibronectin isoform located in the abluminal site of the vessel, could be slow because of interstitial pressure (9). In either case, it is interesting to notice that bivalence and slower clearance of the Ab appear to drive the targeting process, despite the increased Ab size.

The investigation of the role of Ab affinity on tumor targeting performance has been addressed experimentally in the past with binders directed against tumor markers located on the cell membrane (42). Very recently, Adams *et al.* (10) reported the first biodistribution study performed with Ab affinity mutants directed against the same epitope of HER2/*neu*. This more rigorous experimental approach excludes the possibility that differences in targeting performance may be due to different degrees of accessibility of the epitopes within the tumor. Their findings that increased affinity leads to improved selective tumor delivery by monomeric scFvs are in good agreement with our results obtained here with Abs directed against a marker of angiogenesis.

The amount of L19 dimer selectively delivered to the tumor compares favorably with the tumor retention of other recombinant Ab fragments directed against cellular markers assayed in different tumor models in mice (for a review, see Refs. 43, 10, 11, 34, 37, and references therein). Considering that tumoral neovasculature represents only a small percentage of the total tumor mass (Fig. 4), the %ID of L19 retained per gram of vasculature is extremely high and could have an impact in the diagnosis and therapy of tumors with high angiogenic index. It will be interesting to evaluate the usefulness of

L19 for the detection of angiogenesis in patients by immunoscintigraphic (44) and IR fluorescence (12, 45) methodologies, as well as its performance as a selective vehicle for delivering toxic agents to tumor neovasculature (2–8, 46). Work in this direction is in progress in our laboratories.

## Note Added in Proof

Recently, Brekken *et al.* have reported the successful qualitative targeting of tumor angiogenesis using specific monoclonal antibodies against VEGF:receptor complex (47).

## REFERENCES

1. Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.*, 1: 27–31, 1995.
2. O'Reilly, M. S., Holmgren, L., Chen, C., and Folkman, J. Angiostatin induces and sustains dormancy of human primary tumors in mice. *Nat. Med.*, 2: 689–692, 1996.
3. O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. Endostatin: an endogenous inhibitor of angiogenesis and tumour growth. *Cell*, 88: 277–285, 1997.
4. Friedlander, M., Brooks, P. C., Shaffer, R. W., Kincaid, C. M., Varner, J. A., and Chersesh, D. A. Definition of two angiogenic pathways by distinct  $\alpha$ -v integrins. *Science* (Washington DC), 270: 1500–1502, 1995.
5. Pasqualini, R., Koivunen, E., and Ruoslahti, E.  $\alpha$ -v integrins as receptors for tumor targeting by circulating ligands. *Nat. Biotechnol.*, 15: 542–546, 1997.
6. Huang, H., Molema, G., King, S., Watkins, L., Edgington, T. S., and Thorpe, P. Tumor infarction in mice by antibody-directed targeting of tissue factor to tumor neo-vasculature. *Science* (Washington DC), 275: 547–550, 1997.
7. Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S., and Ferrara, N. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. *Nature* (Lond.), 362: 841–844, 1993.
8. Schmidt-Erfurth, U., Diddens, H., Birngruber, R., and Hasan, T. Photodynamic targeting of human retinoblastoma cells using covalent low-density lipoprotein conjugates. *Br. J. Cancer*, 75: 54–61, 1997.
9. Clauss, M. A., and Jain, R. K. Interstitial transport of rabbit and sheep antibodies in normal and neoplastic tissues. *Cancer Res.*, 50: 3487–3492, 1990.
10. Adams, G. P., Schier, R., Marshall, K., Wolf, E. J., McCall, A. M., Marks, J. D., and Weiner, L. M. Increased affinity leads to improved selective tumor delivery of single-chain Fv antibodies. *Cancer Res.*, 58: 485–490, 1998.
11. King, D. J., Turner, A., Farnsworth, A. P. H., Adair, J. R., Owens, R. J., Pedley, R. B., Baldock, D., Proudfoot, K. A., Lawson, A. D. G., Beeley, N. R. A., Millar, K., Millican, T. A., Boyce, B. A., Antoniw, P., Mountain, A., Begent, R. H. J., Schocot, D., and Yarranton, G. T. Improved tumor targeting with chemically cross-linked recombinant antibody fragments. *Cancer Res.*, 54: 6176–6185, 1994.
12. Neri, D., Camemolla, B., Nissim, A., Balza, E., Lepini, A., Querzè, G., Pini, A., Tarli, L., Halin, C., Neri, P., Zardi, L., and Winter, G. Targeting by affinity-matured recombinant antibody fragments of an angiogenesis associated fibronectin isoform. *Nat. Biotechnol.*, 15: 1271–1275, 1997.
13. Zardi, L., Camemolla, B., Siri, A., Petersen, T. E., Paoletti, G., Sebastio, G., and Baralle, F. E. Transformed human cells produce a new fibronectin isoform by preferential alternative splicing of a previously unobserved exon. *EMBO J.*, 6: 2337–2342, 1987.
14. Camemolla, B., Balza, E., Siri, A., Zardi, L., Nicotra, M. R., Bigotti, A., and Natali, P. G. A tumor-associated fibronectin isoform generated by alternative splicing of messenger RNA precursors. *J. Cell Biol.*, 108: 1139–1148, 1989.
15. Castellani, P., Viale, G., Dorcarrato, A., Nicolò, G., Kaczmarek, J., Querzè, G., and Zardi, L. The fibronectin isoform containing the ED-B oncofetal domain: a marker of angiogenesis. *Int. J. Cancer*, 59: 612–618, 1994.
16. Kaczmarek, J., Castellani, P., Nicolò, G., Spina, B., Alemanni, G., and Zardi, L. Distribution of oncofetal fibronectin isoforms in normal, hyperplastic and neoplastic human breast tissues. *Int. J. Cancer*, 58: 11–16, 1994.
17. Camemolla, B., Neri, D., Castellani, P., Lepini, A., Neri, G., Pini, A., Winter, G., and Zardi, L. High-affinity human recombinant antibodies to the oncofetal angiogenesis marker fibronectin ED-B domain. *Int. J. Cancer*, 68: 397–405, 1996.
18. Pini, A., Viti, F., Santucci, A., Camemolla, B., Zardi, L., Neri, P., and Neri, D. Design and use of a phage-display library: human antibodies with subnanomolar affinity against a marker of angiogenesis eluted from a two-dimensional gel. *J. Biol. Chem.*, 273: 21769–21776, 1998.
19. Neri, D., Petrucci, H., Light, Y., Marais, R., Britton, K. E., Winter, G., and Creighton, A. M. Radioactive labeling of recombinant antibody fragments by phosphorylation using human casein kinase II and [ $\gamma$ - $^{32}\text{P}$ ]-ATP. *Nat. Biotechnol.*, 14: 385–390, 1996.
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1990.
21. Munro, S., and Pelham, H. R. B. An Hsp-like protein in the ER: identity with the 78 kD glucose regulated protein and immunoglobulin heavy chain binding protein. *Cell*, 46: 291–300, 1986.
22. Jönsson, U., Fagerström, L., Ivarsson, B., Johnsson, B., Karlsson, R., Lundh, K., Löfås, S., Persson, B., Roos, H., Rönnerberg, L., Sjölander, S., Stenberg, E., Ståhlberg, Urbaniczky, C., Östlin, H., and Malmqvist, M. Real-time bispecific interaction analysis using surface plasmon resonance and a sensor chip technology. *Biotechniques*, 11: 620–627, 1991.
23. Winter, G., Griffiths, A. D., Hawkins, R. E., and Hoogenboom, H. R. Making antibodies by phage display technology. *Annu. Rev. Immunol.*, 12: 433–455, 1994.

24. Figini, M., Marks, J. D., Winter, G., and Griffiths, A. D. *In vitro* assembly of repertoires of antibody chains on the surface of phage by renaturation. *J. Mol. Biol.*, 239: 68-78, 1994.
25. Jespers, L. S., Roberts, A., Mahler, S. M., Winter, G., and Hoogenboom, H. R. Guiding the selection of human antibodies from phage display repertoires to a single epitope of an antigen. *BioTechnology*, 12: 899-903, 1994.
26. Low, N., Holliger, P., and Winter, G. Mimicking somatic hypermutations: affinity maturation of antibodies displayed on bacteriophage using a bacterial mutator strain. *J. Mol. Biol.*, 260: 359-368, 1996.
27. Yang, W., Green, K., Pinz-Sweeney, S., Briones, A. T., Burton, D. R., and Barbas, C. F., III. CDR walking mutagenesis for the affinity maturation of a potent human anti-HIV-21 antibody into the picomolar range. *J. Mol. Biol.*, 254: 392-403, 1995.
28. Schier, R., Balint, R. F., McCall, A., Apell, G., Larrick, J. W., and Marks, J. D. Identification of functional and structural amino acid residues by parsimonious mutagenesis. *Gene (Amst.)*, 169: 147-155, 1996.
29. Huston, J. S., Levinson, D., Muggett, H. M., Tai, M. S., Novotny, J., Margolies, M. N., Ridge, R. J., Brucoleri, R. E., Haber, E., Crea, R., Oppermann, H. Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*, 85: 5879-5883, 1988.
30. Bird, R. E., Hardman, K. D., Jacobson, J. W., Johnson, S., Kaufman, B. M., Lee, S. M., Lee, T., Pope, S. H., Riordan, G. S., and Whitlow, M. Single-chain antigen-binding proteins. *Science (Washington DC)*, 242: 423-426, 1988.
31. Holliger, P., Prospero, T., and Winter, G. "Diabodies": small bivalent and bispecific antibody fragments. *Proc. Natl. Acad. Sci. USA*, 90: 6444-6448, 1993.
32. Neri, D., Montigiani, S., and Kirkham, P. M. Biophysical methods for the determination of antibody-antigen affinities. *Trends Biotechnol.*, 14: 465-470, 1996.
33. Crothers, D. M., and Metzger, H. The influence of polyvalency on the binding properties of antibodies. *Immunochemistry*, 9: 341-357, 1972.
34. Adams, G. P., McCartney, J. E., Tai, M. S., Oppermann, H., Huston, J. S., Stafford, W. F., Bookman, M. A., Fand, I., Houston, L. L., and Weiner, L. M. Highly specific *in vivo* tumor targeting by monovalent and divalent forms of 741F8 anti-c-erbB-2 single chain Fv. *Cancer Res.*, 53: 4026-4034, 1993.
35. Salacinski, P. R. P., McLean, C., Sykes, J. E. C., Clement-Jones, V. V., and Lowry, P. J. Iodination of proteins, glycoproteins and peptides using a solid-phase oxidizing agent, 1,3,4,6-tetrachloro-3 $\alpha$ ,6  $\alpha$ -diphenyl glycoluril (Iodogen). *Anal. Biochem.*, 117: 136-146, 1981.
36. Wu, A. M., Chen, W., Raubitschek, A., Williams, L. E., Neumaier, M., Fischer, R., Hu, S., Odom-Maryon, T., Wong, J. Y. C., and Shively, J. E. Tumor localization of anti-CEA single-chain Fvs: improved targeting by non-covalent dimers. *Immunotechnology (Shannon)*, 2: 21-36, 1996.
37. Rowlinson-Busza, G., Deonarain, M. P., and Epenetos, A. A. Comparison of intact monoclonal antibodies, its F(ab')<sub>2</sub> and Fab fragments and recombinant scFv in a human tumour xenograft model. *Tumor Targeting*, 2: 37-48, 1996.
38. Carter, P., Kelley, R. F., Rodrigues, M., Snedecor, B., Covarrubias, M., Velligan, M. D., Wong, W. L. T., Rowland, A. M., Kots, C. E., Carver, M. E., Yang, M., Bourell, J. H., Shephard, H. M., and Henner, D. High level *Escherichia coli* expression and production of a bivalent humanized antibody fragment. *BioTechnology*, 10: 163-167, 1992.
39. Pack, P., Kujau, M., Schroeckh, V., Knüpfer, U., Wenderoth, R., Riesenberger, D., and Plückthun, A. Improved bivalent miniantibodies, with identical avidity as whole antibodies, produced by high cell density fermentation of *Escherichia coli*. *BioTechnology*, 11: 1271-1277, 1993.
40. Ridder, R., Schmitz, R., Legay, F., and Gram, H. Generation of rabbit monoclonal antibody fragments from a combinatorial phage display library and their production in the yeast *Pichia Pastoris*. *BioTechnology*, 13: 255-260, 1995.
41. Casey, J. L., Keep, K. A., Chester, L., Robson, L., Hawkins, R. E., and Begent, R. H. Purification of bacterially expressed single chain Fv antibodies for clinical applications using metal chelate chromatography. *J. Immunol. Methods*, 179: 105-116, 1995.
42. Colcher, D., Minelli, F. M., Roselli, M., Muraro, R., Simpson-Milenic, D., and Schlom, J. Radioimmunolocalization of human carcinoma xenografts with B72.3 second generation monoclonal antibodies. *Cancer Res.*, 48: 4597-4603, 1988.
43. Kosmas, C., Linardou, H., and Epenetos, A. A. Advances in monoclonal antibody tumour targeting. *J. Drug Target.*, 1: 81-91, 1993.
44. Begent, R. H. J., Verhaar, M. J., Chester, K. A., Green, A. J., Napier, M. P., Hope-Stone, L. D., Cushen, N., Keep, P. A., Johnson, C. J., Hawkins, R. E., Hilson, A. J. W., and Robson, L. Clinical evidence of efficient tumor targeting based on single-chain Fv antibody selected from a combinatorial library. *Nat. Med.*, 2: 979-984, 1996.
45. Folli, S., Westermann, P., Braichotte, D., Pelegrin, A., Weignieres, G., van der Bergh, H., and Mach, J. P. Antibody-indocyanin conjugates for immunophotodetection of human squamous cell carcinoma in nude mice. *Cancer Res.*, 54: 2643-2649, 1994.
46. Neri, D., and Zardi, L. Affinity reagents against tumor-associated extracellular molecules and new-forming vessels. *Adv. Drug Delivery Rev.*, 31: 43-52, 1998.
47. Brekken, R. A., Huang, X., King, S. W., and Thorpe, P. E. Vascular endothelial growth factor as a marker of tumor endothelium. *Cancer Res.*, 58: 1952-1958, 1998.